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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/60, 1/15, 5/14, A01H 5/00

A1

(11) International Publication Number: WO 99/43833

(43) International Publication Date: 2 September 1999 (02.09.99)

(21) International Application Number: PCT/IB98/00232

(22) International Filing Date: 26 February 1998 (26.02.98)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: DISEASE RESISTANT TRANSGENIC PLANTS

(57) Abstract

A method for stimulating the defense response and the disease resistance in plants, particularly potatoes, by increasing the intracellular pyruvate decarboxylase level, as well as a method for producing plants able to express an enhanced level of pyruvate decarboxylase and respective plants etc. are described.

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Disease resistant transgenic plants

Technical Field

The present invention relates to plants and methods for their production, said plants being disease resistant due to transformation with pyruvate decarboxylase (PDC) encoding nucleic acid sequences. Preferred plants are those of the solanaceae family, and within said family preferred plants are potatoes. The disease resistance comprises resistance to *Phytophtora infestans* and resistance to the potato virus Y.

Background Art

The plants' defense response to incompatible pathogen interactions is often manifested as rapid localized host cell death termed the hypersensitive response (HR). Due to said pathogen interaction, a selected group of plant cells die rapidly in the hypersensitive response process. HR is thought to contribute to the containment of the pathogen and is associated with most but not all incompatible host-pathogen interactions and disease resistance (Dangl et al., 1996; Hammond-Kosack and Jones, 1996). During the HR, a battery of defense reactions are initiated by the plant. These include: a burst of reactive oxygen species (Levine et al., 1994; Lamb and Dixon, 1997; Pennell and Lamb, 1997), ion fluxes and activation of H^+/K^+ exchange (Atkinson et al., 1990), callose and lignin deposition and cell wall cross-linking (Bradley et al, 1992; Brisson et al., 1994), lipid peroxidation (Keppler and Baker, 1989), production of antimicrobial phytoalexins (Glazebrook and Ausubel, 1994; Osbourn, 1996), induction of a repertoire of pathogenesis-related (PR) proteins (Bowles, 1990), and disease resistance (see Bent, 1996; Hammond-Kosack and Jones, 1996; Ryals et al., 1996 for reviews). The mechanism of HR is not clear, but it is an active process and involves new transcription and translation by the host (reviewed in Dangl et al.,

1996; Greenberg, 1996). Purified bacterial elicitors can induce HR cell death and disease resistance response when applied locally to a plant (He et al., 1993) indicating that HR is a preset genetic program that can be activated by external factors.

In *Arabidopsis* a number of recessive lesion mimic mutants, called *lsd* (lesion simulating disease) and *acd* (accelerated cell death), have been identified (Dietrich et al., 1994; Greenberg et al., 1994). In maize, more than 32 different loci of both dominant and recessive mutations that form lesions resembling specific pathogen infections have been described (Walbot et al., 1983). For example, the dominant *Les1* and the recessive lethal leaf spot (*lls1*) mutations mimic stereotypic symptoms of *Helminthosporium maydis* and *Helminthosporium carbonum* infections on susceptible maize, respectively (Neuffer and Calvert, 1975; Walbot et al., 1983).

In a number of the lesion mimic mutants, the onset of lesion formation is subject to developmental and environmental changes (Walbot et al., 1983; Dietrich et al., 1994). It has been argued that alteration of cellular homeostasis in such mutants may be misinterpreted by host cells as pathogen infection (Dietrich et al., 1994; Mittler et al., 1995). The connection of altered cellular homeostasis with the activation of PCD is also supported by experiments, in which the ectopic expression of unrelated proteins caused a lesion-phenotype. For example, manipulation of the ubiquitin-dependent protein degradation system (Becker et al., 1993), expression of the bacterio-opsin (bO) proton pump in tobacco (Mittler et al., 1995) and in potato (Abad et al., 1997), and targeting yeast invertase to the apoplast and vacuole of tobacco (Herbers et al., 1996a) were shown to exhibit the lesion mimic phenotype. In these examples, not only lesion formation but also the biochemical markers and disease resistance responses were typical of incompatible host-pathogen interactions and HR cell death.

In early experiments, pathogen infection was shown to increase the tissue sugar levels (Watson and Watson, 1951; Hall and Loomis, 1972), and this increase was correlated with resistance to a pathogen attack (Horsfall and Dimond, 1957). Sugar modulated gene expression in plants is thought to be an adaptive response to developmental and environmental changes (see Koch, 1996 for review). Among the sugar inducible genes are a number of the pathogenesis-related proteins (Johnson and Ryan, 1990; Tsukaya et al., 1991; Herbers et al., 1995, 1996b).

The goal of the present invention was to provide a method for stimulating defense response in plants leading to disease resistance, as well as disease resistant plants.

Disclosure of the invention

It was now surprisingly found that pyruvate decarboxylase, for example such of bacterial origin, like the one described by Conway T. et al. (1987), stimulates the defense response of plants transformed with the respective gene.

Thus one subject of the present invention is a method for stimulating the defense response of plants and plant cells leading to disease resistance, by increasing the average pyruvate decarboxylase level.

Another subject of the present invention is to provide a method for producing a plant or plant cell or reproduction material of said plant comprising a pyruvate decarboxylase encoding DNA sequence incorporated in the genome of said plant, plant cell or reproduction material in a non-natural environment allowing the expression of said pyruvate decarboxylase.

These and other subjects are defined in the independent claims with specific embodiments being disclosed in the dependent claims.

Plants transformed according to the present invention showed lesions of different severity dependent on the amount of transgenic pyruvate decarboxylase production. (The term "transgenic" as it is used herein does not only refer to plants or plant cells or reproduction material or pyruvate decarboxylase production comprising or induced by a heterologous gene, but also by a homologous gene in a non-natural environment. However, due to possible down-regulation rather than overexpression of the target gene in the case of homologous genes, heterologous genes are preferred.) It was found that the defense response was induced in the transgenic plants, even in the absence of visible lesions.

Such transgenic plants, even those with little or no visible lesion development showed markedly enhanced resistance against pathogen infection, as for example infection with bacteria such as *Erwinia*, *Pseudomonas*; fungi such as *Fusarium*, *Alternaria*, *Phytium*, Phytophtora and in particular *Phytophtora infestans*; viruses such as Potexvirus, e.g. Potato Virus X and Potyvirus, in particular Potato Virus Y; mycoplasmas; nematodes; insects. For example a 10 to 300 fold decrease in the number of *Phytophtora infestans* sporangia was found compared to the wild-type. Also an infection with viruses such as the potato virus Y (PVYO803) showed a resistance of transgenic plants.

Due to the fact that the transgenic plants comprising the pyruvate decarboxylase gene have a more or less strong tendency to form lesions, it is preferred that the pyruvate decarboxylase gene is present in a form providing minimal pyruvate decarboxylase related lesions with maximum pathogen resistance under the desired or most likely environmental conditions.

It could be shown that the lesion formation - if observable - with the pyruvate decarboxylase gene from *Zymomonas mobilis* under the control of the 35 S promoter (the gene with the respective promoter was described by

Bucher M. et al. (1994)), usually starts at the tip of fully expanded source leaves and spreads in all directions, primarily along the midrib and veins. Said lesion formation was found to be developmentally and environmentally controlled. At 18°C, in potatoes, the lesions developed 4 weeks after planting in soil, but this phenotype was completely masked by growth at 25°C. The lesions found with the plants according to the invention described here resemble the propagation class lesion mimic mutants, such as the *lls1* of maize (Gray et al., 1997), the *lsd1* and *acd2* of *Arabidopsis* (Dietrich et al., 1994; Greenberg et al., 1994) in the sense that lesion spread was uncontrolled once initiated in responsive cells. The transgenics usually produce higher (generally about 5 to 12-fold higher) levels of acetaldehyde and they usually export a higher (generally about 2 to 10-fold higher) level of sucrose from the leaves compared to the wild-type. Starch content, on the contrary, can be drastically reduced in the high level PDC expressing plants such as potatoes. Analysis of physiological and molecular markers of hypersensitive cell death revealed that defense reactions similar to the HR were initiated in the potato transgenic plants. These included: deposition of callose in the leaf tissue, high level induction of pathogenesis-related (PR) protein encoding transcripts, and heightened resistance to pathogens such as fungus or viruses, e.g. *P. infestans* and potato virus Y.

The present invention is not limited to a specific pyruvate decarboxylase, e.g. of *Zymomonas mobilis* nor to specific plants, although potatoes - because of their great relevance as nutrient - are preferred plants. For the purposes of the present invention any pyruvate decarboxylase encoding DNA sequence with the ability to enhance the naturally present pyruvate decarboxylase level (without killing the plant) is suitable. Suitable DNA sequences are e.g. all sequences encoding a pyruvate decarboxylase or a fragment or mutation thereof

having similar biological activity at least with regard to the effects described herein. Such DNA sequences can be naturally occurring genomic sequences or cDNA sequences, sequences that are identical with such sequences but for the degeneracy of the genetic code as well as sequences encoding fragments and mutations provided that they stimulate defense response. Said encoding sequences can either be introduced into a suitable environment of the genome of the plant to be transformed enabling the expression of pyruvate decarboxylase or they can be introduced into the genome of the plant together with their natural or adapted regulatory sequences. The nucleotide sequence can be introduced into the plant, plant cells, parts of plants such as tissue or reproduction material by any transformation method leading to incorporation of the nucleic acid sequence into the genome of the plant, plant cells, plant tissue or reproduction material. Reproduction materials include stems, tubers, leafs, and cells, with tubers being preferred for potatoes, but also calli.

Although it is by no means intended to add any limitation in respect of specific mechanisms, some considerations in view of the results found are made on the initiation of a hypersensitive response by overexpression of PDC.

A first interpretation of the results obtained might be that the response is triggered by an interaction of acetaldehyde with a component of the cell death machinery. In said first scenario, the physical and chemical properties of acetaldehyde will determine its interaction with endogenous molecules. However, potatoes incubated under anoxia to enhance pyruvate decarboxylase activity, and exposed back to air produced high acetaldehyde levels, but no lesions could be observed. Thus, there was no indication that an enhanced pyruvate decarboxylase activity might lead to lesion formation and pathogen resistance. When the acetaldehyde concentrations

of transgenic plants were measured, it was found that acetaldehyde accumulated to higher levels in the transgenics grown at 18°C compared to the wild-type, and that L-8 produced as much acetaldehyde in the 25°C as any other of the transgenic potatoes at the 18°C growth condition. However, the transgenic potatoes showed lesions at 18°C, whereas L-8 at 25°C was virtually unaffected. Also for non-transformed potatoes incubated under anoxia and exposed back to air (see above) it was found that they produced high levels of acetaldehyde equivalent to L-8 under normoxia at 18°C, but no lesions were observed. Thus, another or other factors could also be relevant for the lesion formation and the defense response.

In a second scenario, an unbalanced biochemical state of the cell imposed by transgene expression might be assumed as a first trigger of the defense response. Support for this model stems from the transgenic approach of Mittler et al. (1995), and Becker et al. (1993), where they showed that expression of a bacteriopsin proton pump, and perturbation in protein metabolism caused by alteration of the ubiquitin protein degradation system, respectively lead to the formation of distinct HR-like lesions in transgenic tobaccos. Herbers et al. (1996a) showed that a change in sugar metabolism mediated by the expression of a yeast invertase in tobacco resulted in a lesion mimic phenotype on source leaves. Nothing similar is found in literature for PDC, however data found for plants according to the present invention showed that the total soluble sugar increases approximately up to 2-fold, and the translocated sucrose increases by 2 to 10-fold in the transgenics compared to the wild-type. Increased levels of soluble sugars in the tissue during pathogen infections has long been noted (Watson and Watson, 1951; Hall and Loomis, 1972). It has also been shown that pathogenesis-related proteins such as PAR-1, PR-Q, chalcone synthase, and proteinase inhibitor II were induced by sugars (Johnson and Ryan, 1990;

Tsukaya et al., 1991; Takeuchi et al., 1994; Herbers et al., 1996b). Thus, the surprisingly found influence of the pyruvate decarboxylase expression on sugar and starch levels could be involved in triggering the plant defense response and the disease resistance. As mentioned above, these two possibilities are not mutually exclusive, and therefore shall not be regarded as limiting the scope of the invention. Alternative possibilities might e.g. be a direct relationship between carbohydrate metabolism, oxidative stress and cell death.

In any case, an increased amount of pyruvate decarboxylase in the cell in comparison with the wild type over a sufficient long time to activate defense response is likely to be the main factor. Thus, any other method to increase said amount is also within the scope of the present invention, i.e. the application of pyruvate decarboxylase in any kind of carrier enabling the introduction of PDC into plant cells.

For obvious reasons it is desired that by the incorporation of the pyruvate decarboxylase gene the lesion formation is largely reduced to ensure a good harvest with at the same time enhanced pathogen resistance. This can e.g. be obtained by selection of the minimal lesion forming plants showing resistance or by variation of the promoter, e.g. by selecting a promoter that needs specific activation by a specific inducer.

Brief Description of the drawings

Figure 1 shows the *Zymomonas* PDC expression and *in vitro* enzymatic activity, with

Figure 1A representing the *Zymomonas* PDC protein expression in potato leaves, whereby twenty micrograms of total soluble protein were loaded per lane and probed with anti-*Zymomonas* PDC antibody, and

Figure 1B representing the PDC enzymatic activity, whereby the PDC activity was measured in the leaf

total soluble protein extract in an ADH coupled spectrophotometric assay, with one unit converting 1µmol of pyruvate into acetaldehyde per minute. L, transgenic line; wt, wild-type.

Figure 2 shows the acetaldehyde concentration in potato leaf tissue, with

Figure 2A showing chromatograms of control experiments, with the peaks representing: blk, water blank; con, control in which the wild-type leaf extract was incubated at 37°C for 1 h; wt, wild-type leaf untreated; anx, wild-type leaf incubated under anoxia for 2 h and 10 min air; std, acetaldehyde standard (1µM), and

Figure 2B representing the acetaldehyde from plant leaves grown at 18±3°C, and

Figure 2C representing the acetaldehyde from plant leaves grown at 25±2°C, whereby the acetaldehyde was measured as a fluorescent adduct with 1,3-cyclohexanedione in a perchloric acid extract of 4 week old leaves by HPLC, and whereby the values represent the mean ± SE of 6 independent measurements.

Figure 3 shows the accumulation of PR gene transcripts in transgenic potato leaves, whereby total RNA (10 µg) from healthy (-lesion) or lesioned (+lesion) leaves of line 25 was loaded per lane and probed with specific cDNA probes indicated on the left side.

Figure 4 shows soluble sugars and starch in potato leaves, whereby the values represent the mean ± SE of 5 measurements of individual plants, and whereby

Figure 4A represents the concentration of starch and soluble sugars in the leaf tissue from plants grown at 18±3°C,

Figure 4B represents the concentration of soluble sugars in the petiole exudates of plants grown at 18±3°C, and

Figure 4C represents the concentration of soluble sugars in the petiole exudates of plants grown at 25±2°C.

Figure 5 shows resistance tests to the fungus *Phytophtora infestans*, particularly the sporulation efficiency 6 days post-infection, whereby the values represent the mean ± SE of 3 leaves of 8 individual plants for each line.

Figure 6 shows resistance tests to the PVY, particularly the virus titer measured by ELISA in three different wt and transgenic (L-17) plants.

Modes for Carrying out the Invention

Example 1: Transformation of potato plants with Zym PDC and expression of ZymPDC

The PDC gene from the obligate anaerobe *Zymomonas mobilis* (Conway T., et al. (1987)), was inserted between the alfalfa mosaic virus (AMV) translational enhancer and the nos terminator under the control of the 35S promoter (Odell J.T. et al., (1985)) as described in Bucher et al., (1994).

The cloning of the *Zymomonas mobilis* PDC gene in pMON505 expression vector under the control of the CaMV 35S promoter was described previously (Bucher et al., 1994). Potatoes were transformed using the *Agrobacterium* system according to Rocha-Sosa et al. (1989) and propagated from tissue cultures.

Potato plants (*Solanum tuberosum* var. Désirée) were grown in a greenhouse or growth room at 16/8 h light/dark cycle and a temperature of either 18±3°C or 25±2°C.

Northern analysis, Western bloting, and PDC enzymatic assay were made essentially as described before (Bucher et al., 1994).

Transgene expression was variable and 4 lines accumulating the PDC protein to different levels (Figure 1A) were maintained in tissue culture by clonal propagation. The *in vitro* PDC enzymatic activity correlated with the level of protein accumulation, and showed more than a 6-fold increase in the case of highest expression in line 8 (L-8) as compared to the wild-type (wt) (Figure 1B).

The transgenic plants displayed a lesion mimic phenotype, the severity of which correlated with the expression level of the *Zym*PDC protein, the highest expressors showing the most extensive lesions. Lesions began to develop about 4 weeks after planting in soil. In most cases, the lesion started at the tip of source leaves near the midrib as small brownish spots, and spread in all directions, primarily along the midrib and the veins. Within 3 to 5 days of the start of lesion formation, the lesion encompassed most of the leaf area leading to a dry, grey to brownish, and shrunken appearance. Finally, the whole leaf collapsed and abscised. The lesion continued to the next fully expanded source leaf, and proceeded to the one above even before the complete collapse of the one below it. The timing and progression of the lesion showed dependence on the level of the transgene expression. The lines that expressed the PDC protein at highest levels were the first to show the lesion appearance. The difference in symptom appearance between the highest expressor (L-8) and the least expressors (L-21 and L-17) was at least 6-10 days. The progression of the lesion was also dependent on the PDC protein levels. In L-8 and L-25, the lesion spread was fast and uncontrolled, leading to the death of the entire plant within 2-3 weeks after the onset of lesion formation. In L-17 and L-21, the lesions were localized, spread slowly to consume the entire leaf, but remained restricted to only a few source leaves, and never reached the top of the plant. The transgenic plants were not reduced in height before lesion formation, and L-17 and L-21 grew to

a similar height as the wild-type even after lesion development.

Example 2: Environmental influence

Plants as described under Example 1 were grown at different temperatures and humidities. The respective studies showed that the phenotypes investigated were reproducible in all seasons when potatoes were grown at a temperature of 18±3°C in greenhouses or growth rooms. However, when plants were grown at a temperature of 25±2°C, the lesion phenotype was masked and all the transgenics grew to full maturity. Occasionally, localized lesions appeared on one or two leaves of L-8, but these were weaker in magnitude than the lesions of L-17 and L-21 at 18°C, and in most cases did not spread to engulf the entire leaf. When plants were transferred from 18 to 25°C after lesion development, further lesion progression stopped and the transgenics resumed normal growth. Even the highest expressor, severely affected L-8, fully recovered when transferred to 25°C. In this case, the recovery required 1-2 days, and newly developing leaves and shoots could be observed within a week. When plants that had been maintained at 25°C for 4 weeks were transferred to the 18°C growth condition, the lesion started within 2-3 days in the case of L-8 and L-25, and after one week in the case of L-17 and L-21.

Example 3: Acetaldehyde level in leaf tissue of transgenic potatoes

PDC is known to catalyze the first step in ethanolic fermentation, a decarboxylation of pyruvate yielding acetaldehyde and CO_2. In wild-type leaves, ethanolic fermentation occurs only during oxygen limitation and some other stresses. An attempt for constitutive high level expression of bacterial PDC in tobacco leaves did neither lead to measurable acetaldehyde production nor to visible lesion formation in the presence of oxygen as de-

termined by gas chromatography (Bucher et al., 1994). The techniques for the detection of acetaldehyde in plants have been restricted to gas chromatography and enzymatic methods, both of which are unable to detect low level tissue concentrations. Since the transgenic potato plants analysed here developed lesions under normoxic conditions, and the severity of the lesion correlated with the level of transgene expression, the determination of the acetaldehyde levels was desired. Therefore a sensitive high-performance liquid chromatography (HPLC) method (Helander et al., 1993), in which acetaldehyde is measured as a fluorescent adduct with 1,3- cyclohexanedione and ammonium ion was adapted as follows:

Healthy leaf discs (2 per plant) were snap frozen in liquid nitrogen from leaf number 3 and 4 as counted from the top of a 4 week old potato. The leaves were extracted with 6% perchloric acid at 4°C and incubated on ice for 2 h. After incubation, the samples were spun at maximum microfuge speed for 10 min at 4°C. The supernatant was neutralized to pH 6.0-6.5 with 5M K_2CO_3 on ice. The neutralized extract was spun again for 10 min as above, and the supernatant was transferred to a precooled Eppendorf and kept on ice until derivatization. Acetaldehyde in this extract was measured as a fluorescent adduct formed by a reaction with 1,3-cyclohexanedione (CHD) essentially according to Helander et al. (1993). The reaction mixture contained 150 µl ammonium acetate (20%,w/v, in water), 150 µl thiourea (6%, w/v, in water), 50 µl CHD 1.25%, w/v, in water), and 150 µl extract added to a 2 ml glass bottle in this order. Each bottle was immediately sealed after adding the extract, and all samples were incubated at 60°C in a gently shaking water both for 1 h. The samples were cooled on ice and 20 µl aliquots were analyzed by high- performance liquid chromatography (HPLC). The HPLC system set up was as described in Helander et al. (1993) with the following modifications: System Gold HPLC System (Beckmann, Nyon, Switzerland), a

Rheodyne RH 7010 injector with a 100 µl sample loop (Beckmann, Nyon, Switzerland), and Nucleosil 100-5 C18 reversed-phase analytical column (40 × 250 mm i.d., 5 µm particle size; Macherey-Nagel, Oensingen, Switzerland) were used. The column was eluted isocratically at a flow rate of 1.0 ml/min at ambient temperature with a mobile phase consisting of methanol-water (40: 60, v/v). A Kontron Model SFM-25 fluorescent detector (Kontron, Zurich, Switzerland) was used with excitation and emission wavelengths of 366 and 440 nm, respectively.

The volatility of acetaldehyde was used to authenticate the assay conditions. As negative controls extractions were performed at room temperature and the extracts were incubated at 37°C for 1 h before mixing with cyclohexane. In this control, as well as in the water blank, a small peak with a comparable height appeared with the same retention time as the acetaldehyde adduct. This was considered to represent the background peak. As positive controls, wild-type potato leaves were incubated for 2 h under anoxia and 10 min in air before extraction, where more than a 10-fold increase in the acetaldehyde peak was observed compared to the untreated wild-type.

This method allowed the determination of low levels of acetaldehyde in actively respiring normoxic potato leaves (see Figure 2).

Figure 2A shows chromatograms of control experiments used to authenticate the assay conditions. In the water blank and wild-type leaf extract incubated at 37°C for 1 h, a small background peak appeared which has the same retention time as the acetaldehyde adduct. A similar background peak was observed by Helander et al. (1993). As positive controls, wild-type potato leaves were incubated under anoxia, in which more than a 10-fold increase in acetaldehyde peak area was observed compared to the untreated wild-type (Figure 2A). In wild-type potato leaves a low but significant amount of acetaldehyde was measured at both 18 and 25°C growing conditions

(Figures 2B and C). When the transgenics were grown at 18°C, a 5 to 12-fold increase was measured compared to the wild-type (Figure 2B). This increase in tissue acetaldehyde content was related to the level of PDC protein, the highest being detected in L-8 (Figure 2B). At 25°C the level of acetaldehyde was drastically reduced in all the transgenics, only in L-25 and L-8 were the values significantly higher than in the wild type (Figure 2C). Interestingly, a comparison between acetaldehyde levels at 18 and 25°C showed that there was no absolute correlation between acetaldehyde levels and lesion formation. For instance, L-21 at 18°C and L-8 at 25°C have comparable acetaldehyde levels, yet L-21 developed severe symptoms at 18°C, whereas L-8 at 25°C was virtually unaffected. This clearly indicates that in addition to acetaldehyde concentration other physiological and environmental factors must contribute to lesion formation.

Example 4: Correlation between lesion formation and induction of the plant defense response

Since the lesions resembled those occurring during pathogen infection, it was investigated whether classical defense reactions were initiated in transgenic potatoes. Callose deposition represents one of the earliest plant defense responses (Bradley et al., 1992), although it is not an exclusive marker of HR.

Callose deposition was detected as follows: Leaf discs for callose examination were bleached in a series of 50, 75 and 96% ethanol overnight. The cleared leaves were rinsed in water and stained for 1 h at room temperature in a humid chamber in a 0.05% (w/v) solution of aniline blue in 0.15 M K_2HPO_4. Stained leaves were examined under UV-light using excitation filter, 365 nm; dichromic mirror, 396 nm; and barrier filter, 420 nm.

After treatment with aniline blue fluorescence could be observed in transgenic leaves.

The intensity of the fluorescence was stronger with the higher expressors of PDC protein, and the highest deposition of callose was observed within and surrounding the lesion area.

In response to pathogens, plants also induce specific antifungal component enzymes such as β-1,3-glucanases and chitinases (Mauch et al., 1988; Zhu et al., 1994), also known as pathogenesis-related (PR) proteins. The mRNA levels of three typical potato PR genes (gst1, glucanase, and chitinase) were increased by 8 up to about 45-fold in the transgenics compared to the wild-type (Figure 3). Lesion formation enhanced the accumulation of the mRNAs, but healthy leaves also showed significantly higher level of PR gene induction as compared to the wild-type (Figure 3). These results show the activation of multiple defense responses in the transgenic plants.

Example 5: Correlation of lesion formation and major changes in sugar metabolism

Due to the fact that the lesion phenotype starts in the fully expanded source leaves and progresses towards the sink it was assumed that possibly sugar metabolism might be involved in the process. Therefore the sugar and starch content in the fully expanded source leaves at 18°C was measured as follows:

Leaf discs (2 per plant) were collected in liquid nitrogen from fully expanded source leaves after 8h of the light period. Most of the L-8 and L-25 plants growing at the 18°C showed lesions at the time of sampling (4-5 weeks after planting to soil), but samples were collected from healthy looking leaves. Leaves were homogenised in an Eppendorf tube with 80% ethanol (v/v) and extracted for 90 min at 70°C. Samples were spun at 13 000 rpm for 10 min, and the supernatant was stored at - 20°C for soluble sugars (sucrose, glucose, and fructose) determination. The pellet was washed with 1 ml of

80% ethanol twice and spun in the same way. The washed pellet was resuspended in 400 µl of 0.2 M KOH and incubated at 95°C for 1 h. The starch was solubilised further at 50°C overnight. The volume was adjusted to the 0.5 ml mark with KOH, neutralized with 1 M acetic acid, and centrifuged at 13 000 rpm for 10 min. This supernatant was used to determine starch content. Sucrose, glucose, fructose, and starch were measured enzymatically using a Boehringer kit (Mannheim, Germany).

Figure 4 shows the amount of soluble sugars and starch at the initiation of lesion formation. A dramatic decrease of starch content in the highest PDC expressors, L-25 and L-8, was found compared to the untransformed wild-type (Figure 4A). The soluble sugars, on the other hand, were found to be somewhat elevated in the transgenics compared to the wild-type (Figure 4A). However, this increase was not consistent with the level of transgene expression, and insufficient to account for the drastic drop in starch content.

Since the decrease in starch content was not fully compensated by a corresponding increase in soluble sugar levels, the export from the leaf was examined. Fully expanded source leaves were cut from the base of their petiole, placed in an EDTA solution for 8 h, and soluble sugars were measured in the exudate. At this time the leaves looked completely healthy. The exported sucrose increased by approximately 2 to 10-fold in the transgenics compared to the wild-type (Figure 4B), suggesting that most of the mobilized starch was transported out of the tissue in the form of sucrose. Although glucose and fructose were slightly elevated in the transgenics compared to the wild-type, the values remained low and comparable in all the transgenics (Figure 4B). Thus, transport is specific for sucrose, and unlikely to be a passive response of collapsing and dying cells. Petiole exudates from plants grown at 25°C showed no difference between the transgenics and the wild-type in sucrose ex-

port (Figure 4C). The petiole exudates were determined as follows:

Fully expanded source leaves (1 leaf per plant) from 4-5 week old potatoes were cut from the base of their petiole and immersed immediately into 3 ml of 5 mM EDTA (pH 6.0) solution according to Riesmeier et al. (1994). The petiole exudates were collected for 8 h under the same plant growth condition, and soluble sugars were determined enzymatically as above.

It is not known why sucrose should be exported in the transgenics, but the conversion of starch into sucrose and its correlation with the PDC expression suggests that sucrose translocation is a component of the developmentally regulated cell death initiation and execution process.

Example 6: Phytophtora infestans resistance of transgenic potatoes

The response of the PDC transgenics to a virulent fungal inoculation was examined to determine whether the transgenics exhibit disease resistance.

Transgenic and wild-type potato leaves were infected with the fungal pathogen *Phytophtora infestans*, causative agent of late blight disease, to which the wild-type Désirée variety is susceptible. Both the transgenic and wild-type leaves were completely healthy at the time of infection.

An inoculum of *Phytophtora infestans* strain 94-18 was prepared by adding 15 ml of ice cold 0.5% glucose to a 3 weeks old culture on rye A medium (Ribeiro, 1978). After 3 h incubation at 4°C to allow for the release of the zoospores, spores were counted and the concentration was adjusted to 25,000 per ml. Eight plants per line were grown in the greenhouse for 3 weeks at 24 ± 2°C and transferred to 17°C for the infection test. On each plant, 3 leaves were infected with 4 droplets of 5 µl of spore suspension per leaf. Mock inoculations were

done in the same way, except that 5 µl droplets of 0.5% glucose were spotted on the leaves. Inoculated plants were kept covered to maintain high humidity for 24 h. Plants were covered again 40 h before scoring. Six days after infection, the 3 infected leaves per plant were placed in a 50 ml tube containing 30 ml of 10% ethanol and shaken at 300 rpm for 20 min. Sporulation was estimated by counting the sporangia in the remaining solution.

Lesions spread faster in the transgenics than in the wild-type, the rate of lesion propagation correlating with the level of PDC expression. Spread of the pathogen was assessed after 6 days of infection. More than 90% of the L-8 leaf area was covered by the lesions, whereas in L-25 the proportion of the leaf area covered by the lesions was about 60%. In L-17 leaves, the 4 inoculation spots could be distinguished, but were more irregular and diffuse than the wild-type. In the wild-type leaves, the lesion spread slowly covering less than 20% of the total area and the lesions appeared as 4 distinct areas well separated from one another. The mock inoculated L-25 and wild-type controls on the other hand, showed no lesion at all, but L-8 did show some lesion formation (data not shown), as is expected for this high expressor line. Microscopic counting of fungal sporangia on the leaves after 6 days of infection, on the contrary, showed that the transgenics considerably impaired the fungal propagation. In the transgenics, a 10 to 300-fold decrease in the number of sporangia was found compared to the wild-type (Figure 5). This indicates that the wild-type is much more susceptible to the infection than any of the transgenics.

Example 7: **Resistance test of wild type and transgenic PDC potato plants (line 17) to infection by potato virus Y (PVY 0803).**

Two weeks old wt and transgenic L-17 potato plants (3 plants each) grown at 24 ± 2°C in a green house were inoculated on a young leaf with potato virus Y (PVY0803) by rubbing the leaf with carborendum. The inoculated plants were incubated in a green house at a light/dark cycle (16 hours light, 21 ± 1° C / 8 hours dark, 17 ± 1° C) and symptoms of viral infection were monitored on the plants. The mock inoculated plants did not develop symptoms. At various days after infection, samples from the infected leaf or from an upper leaf (fourth leaf above the infected leaf) were analyzed by ELISA and the virus titer was determined.

Wt plants showed symptoms of viral infection in the infected leaf but also showed PVY symptoms in the upper leaves of the plant. In contrast, the transgenic plants showed symptoms in the inoculated leaf, but no lesions developed in the rest of the plant.

These results were confirmed by ELISA analysis of leaf tissue. The inoculated leaf was found to contain viruses in both the wt and the transgenic plants 13 dpi. In the wt plants, the titer was high in the upper leaf 17 and 20 dpi. However the virus could not be detected in the upper leaves in the transgenic plants (Fig. 6).

There is no progression of lesion formation due to virus infection in the transgenic plants as compared to the wt. The transgenic plants showed a resistance to potato virus Y, both at the macroscopic level, with absence of any visible lesions, and at the ELISA analysis level, where the virus can not be detected in the upper leaf analyzed.

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited

thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

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Claims

1. Process for stimulating the defense response in plant cells and/or plants and/or reproduction material of plants comprising such cells characterized in that said process comprises the step of transforming plant cells, plants, parts of plants or reproduction material of plants with a DNA sequence comprising a pyruvate decarboxylase encoding sequence, whereby said DNA sequence is stably integrated into the genome in an environment enabling the expression of pyruvate decarboxylase in an amount to enhance resistance and, optionally, replicating said plant cells and/or plants and/or reproduction material.

2. Process according to claim 1, wherein the pyruvate decarboxylase encoding DNA sequence is a heterologous sequence.

3. Process according to claim 2 wherein the pyruvate decarboxylase encoding sequence is of bacterial origin.

4. Process according to claim 3 wherein the origin of the pyruvate decarboxylase encoding sequence is *Zymomonas mobilis*.

5. Process according to anyone of claims 1 to 4 wherein the plants are of the Solanaceae family.

6. Process according to claim 5, wherein the plants are potatoes

7. Process according to anyone of claims 1 to 6 wherein the expression is started or enhanced after promoter stimulation.

8. Process according to anyone of claims 1 to 7 wherein the pyruvate decarboxylase expression is started or enhanced after promoter stimulation.

9. The process of anyone of claims 1 to 8 wherein the defense response is stimulated against patho-

gens selected from fungus or viruses, particularly *Phytophtora infestans* or potato virus Y.

10. The process of anyone of claims 1 to 9 wherein the pyruvate decarboxylase encoding sequence is provided with the mosaic virus Ca MV 35 S promoter.

11. The process of claim 10 wherein the transformation is performed using expression vector p MON 505.

12. A process for producing a plant or reproduction material of said plant which is transformed with a DNA sequence comprising a pyruvate decarboxylase encoding sequence and being capable to express pyruvate decarboxylase in said plant or reproduction material, which process comprises transforming cells or tissue of said plants with a DNA sequence comprising a pyruvate decarboxylase encoding sequence thus that the DNA sequence is stably integrated into the genome in an environment enabling the expression of pyruvate decarboxylase in said plant cells or plant tissue, regenerating plants and/or reproduction material of said plants from the plant cells or tissue transformed with said DNA sequence and, optionally, biologically replicating said last mentioned plants or reproduction material or both with the proviso that the plant is not a tobacco plant.

13. Process according to claim 12, wherein the pyruvate decarboxylase encoding DNA sequence is a heterologous sequence.

14. Process according to claim 13 wherein the pyruvate decarboxylase encoding sequence is of bacterial origin.

15. Process according to claim 14 wherein the origin of the pyruvate decarboxylase encoding sequence is *Zymomonas mobilis*.

16. Process according to anyone of claims 12 to 15 wherein the pyruvate decarboxylase encoding DNA sequence is under the control of a promoter which starts or enhances the expression after stimulation.

17. The process of anyone of claims 12 to 16 wherein the defense response is stimulated against pathogens selected from fungus or viruses, particularly *Phytophtora infestans* or potato virus Y.

18. The process of anyone of claims 12 to 17 wherein the pyruvate decarboxylase encoding sequence is provided with the mosaic virus Ca MV 35 S promoter.

19. The process of claim 18 wherein the transformation is performed using expression vector p MON 505.

20. Process according to anyone of claims 12 to 19 wherein the plant is of the Solanaceae family, in particular a potato plant.

21. A plant transformed with a pyruvate decarboxylase encoding DNA sequence which sequence is stably integrated into the genome of the plant in an environment enabling the expression of pyruvate decarboxylase with the proviso that the plant is not a tobacco plant.

22. Plant according to claim 21 which is a plant of the Solanaceae family, in particular a potato plant.

23. A reproduction material of a plant transformed with a pyruvate decarboxylase encoding DNA sequence which sequence is stably integrated into the genome of the plant in an environment enabling the expression of pyruvate decarboxylase, with the proviso that the plant is not a tobacco plant.

24. Reproduction material of a plant according to claim 23 whereby the plant is of the Solanaceae family, in particular a potato plant.

25. Plant cells transformed with a pyruvate decarboxylase encoding DNA sequence which sequence is stably integrated into the genome of the plant in an environment enabling the expression of pyruvate decarboxylase with the proviso that the plant is not a tobacco plant.

26. Plant cells according to claim 25 whereby the plant is of the Solanaceae family, in particular a potato plant.

27. The process for producing a plant or reproduction material of said plant, or the plant, or the reproduction material, or the plant cells of anyone of claims 12 to 26 wherein the plant, reproduction material or plant cells are capable to express pyruvate decarboxylase in an amount sufficient to stimulate defense response against pathogens.

28. Use of pyruvate decarboxylase or pyruvate decarboxylase encoding DNA sequences enabling the expression of pyruvate decarboxylase for activating the defense response of plants, plant cells and/or reproduction material of plants.

29. Use of pyruvate decarboxylase or pyruvate decarboxylase encoding DNA sequences for enhancing the intracellular amount of pyruvate decarboxylase in potato plants, plant cells , reproduction material or plant tissue under normoxic conditions in comparison with the wild type.

30. Process for selectively protecting the culture of a plant transformed with a pyruvate decarboxylase encoding DNA sequence which sequence is stably integrated into the genome of the potato plant and under the control of a promoter enabling the expression of pyruvate decarboxylase after stimulation of said promoter, comprising the step of treating the field with a promoter inducer.

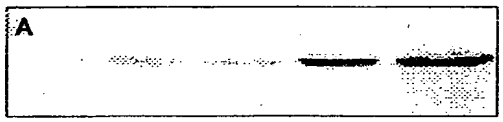

Fig. 1A

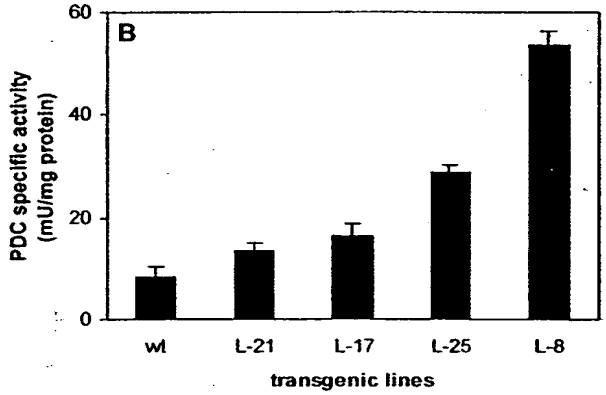

Fig. 1B

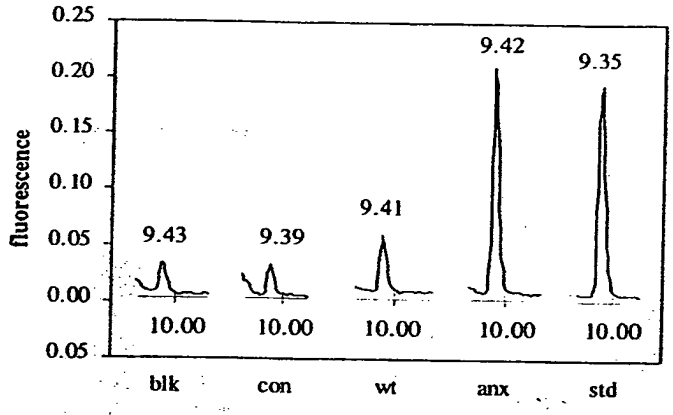

Fig.2A

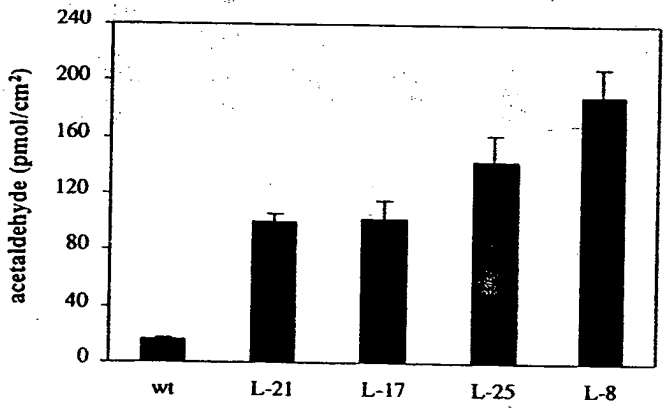

Fig.2B

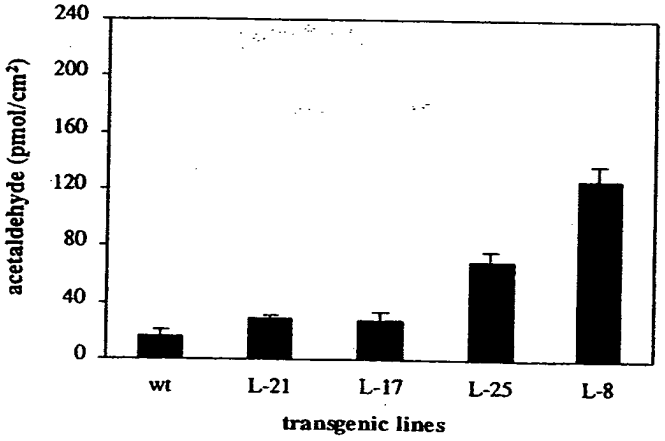

Fig.2C

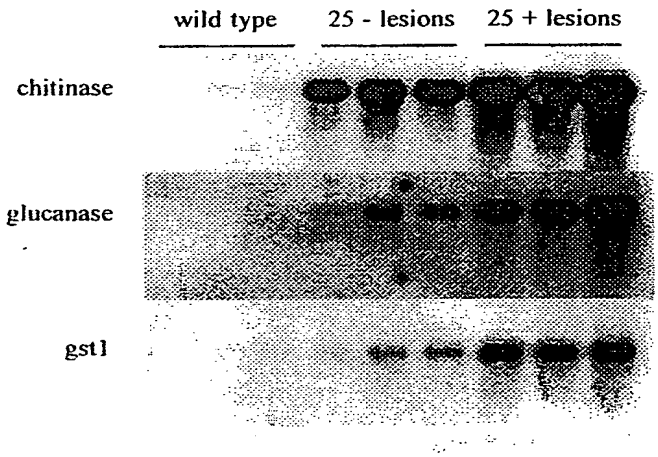

Fig. 3

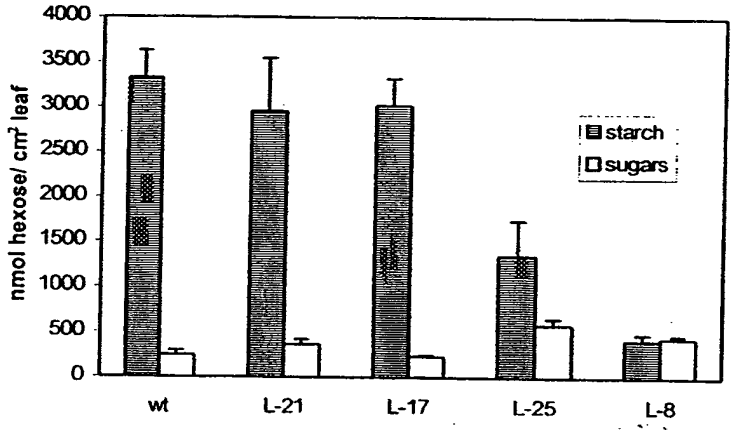

Fig. 4A

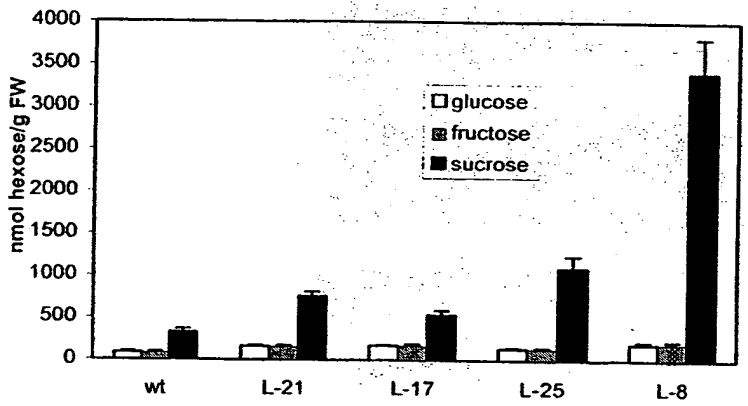

Fig. 4B

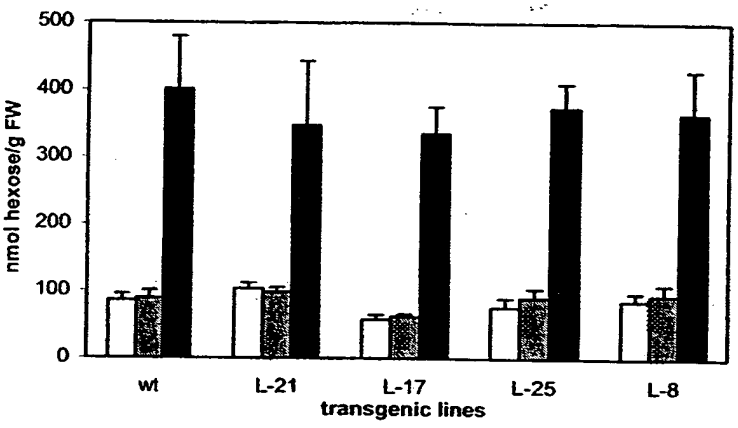

Fig. 4C

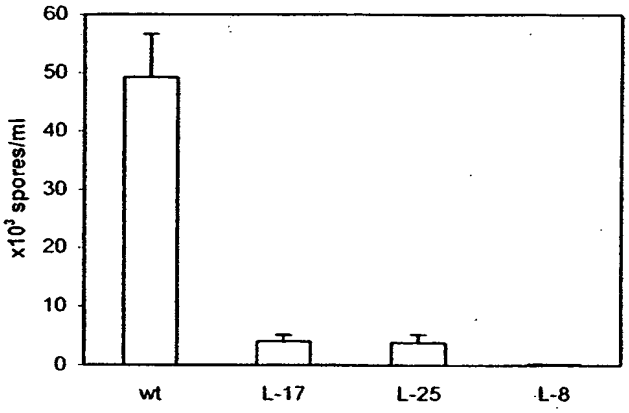

Fig. 5

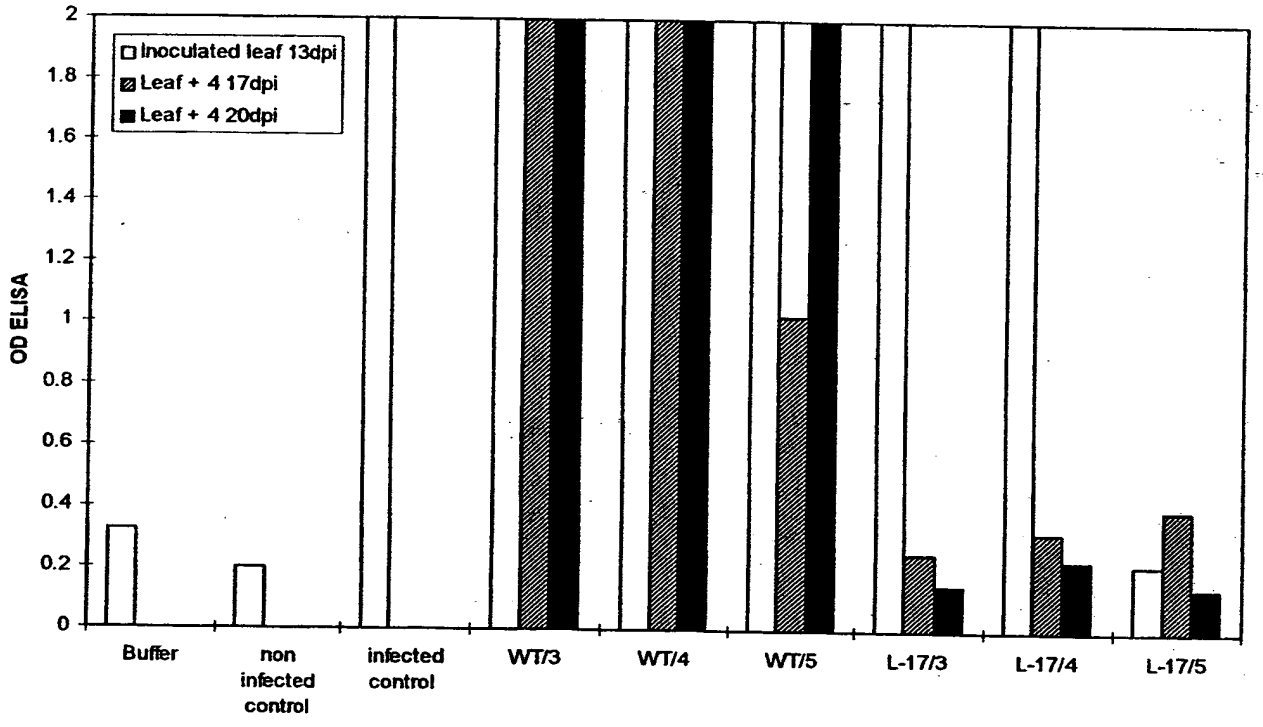
Inoculated leaf 13dpi
Leaf + 4 17dpi
Leaf + 4 20dpi
OD ELISA
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Buffer
non infected control
infected control
WT/3
WT/4
WT/5
L-17/3
L-17/4
L-17/5

Fig. 6

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/IB 98/00232

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/60 C12N1/15 C12N5/14 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 27295 A (HORTICULTURE RESEARCH INTERNAT ;MANNING KENNETH (GB)) 31 July 1997 see the whole document	1-30
X	BUCHER M ET AL: "Ethanolic fermentation in transgenic tobacco expressing *Zymomonas* *mobilis* *pyruvate* *decarboxylase*." EMBO J, JUN 15 1994, 13 (12) P2755-63, ENGLAND, XP002071433 cited in the application see the whole document	1-30

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1998	01/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hillenbrand, G

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/IB 98/00232

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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